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Acetaminophen Induces Apoptosis of C6 Glioma Cells by Activating the c-Jun NH_2-Terminal Protein Kinase-Related Cell Death Pathway

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ABSTRACT

Acetaminophen (AAP), a widely used analgesic drug, can damage various organs when taken in large doses. In this study, we investigate whether AAP causes cell damage by altering the early signaling pathways associated with cell death and survival. AAP caused time- and concentration-dependent apoptosis and DNA fragmentation of C6 glioma cells used as a model. AAP activated c-Jun N-terminal protein kinase (JNK) by 5.3-fold within 15 min. The elevated JNK activity persisted for up to 4 h before it returned to the basal level at 8 h. In contrast, activities of other mitogen-activated protein (MAP) kinases and the level of Akt phosphorylation in the cell survival pathway remained unchanged throughout the treatment. Wortmannin, an inhibitor of phosphatidylinositol-3 kinase, or SB203580, an inhibitor of p38 MAP kinase, did not reduce AAP-induced toxicity, indicating that these enzymes do not play a major role in cell toxicity. AAP-induced apoptosis was preceded by the sequential elevation of the pro-apoptotic Bax protein, cytochrome *c* release, and caspase-3 activity. Treatment with caspase inhibitor benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (Z-DEVD-FMK) significantly reduced AAP-induced caspase-3 activation and cytotoxicity. Transfection of cDNA for the dominant-negative mutant JNK-KR or stress-activated protein kinase kinase-1 Lys→Arg mutant (SEK1-KR), an immediate upstream kinase of JNK, significantly reduced AAP-induced JNK activation and cell death rate. The noncytotoxic analog of AAP, 3-hydroxyacetanilide, neither increased JNK activity nor caused apoptosis. Pretreatment with YH439, an inhibitor of *CYP2E1* gene transcription, markedly reduced CYP2E1 mRNA, protein content, and activity, as well as the rate of AAP-induced JNK activation and cell death. These data indicate that AAP can cause cell damage by activating the JNK-related cell death pathway, providing a new mechanism for AAP-induced cytotoxicity.

Acetaminophen (paracetamol; *N*-acetyl-*p*-aminophenol; AAP) is one of the most frequently used analgesics/antipyretics and is generally considered safe in therapeutic doses. However, when AAP is consumed in large doses, it is known to damage various cells or tissues including hepatic, renal, cardiac, and central nervous system (Thomas, 1993). In severe cases, it can cause death in humans and laboratory animals. Organ toxicity caused by AAP overdose and long-term addictive usage are not rare in humans. For example, more than 70 patients were hospitalized at an urban county hospital over a 40-month period because of excessive AAP ingestion (Schiodt et al., 1997). The extent of AAP-induced toxicity can be exacerbated when the levels of tissue antioxidants such as glutathione are low after long-term alcohol consumption and/or inadequate nutritional intake or fasting (Thomas, 1993; Whitcomb and Block, 1994). Earlier studies suggested that hepatotoxic doses of AAP (350–500 mg/kg) could cause hepatic apoptosis as well as necrosis in laboratory animals (Lawson et al., 1999). AAP-induced toxicity is believed to be mediated through its metabolism primarily by mixed-function oxidases, including cytochromes P450 2E1, 1A2, and 3A in humans and experimental animals (Raucy et al., 1989; Sinclair et al., 2000). Furthermore, it was shown that AAP and other CYP2E1 substrates caused cell death of HepG2 hepatoma or PC12 cells transfected with CYP2E1 cDNA (Dai and Cederbaum, 1995; Holownia et al., 1997; Lin et al., 1999). In contrast, the parent HepG2 or PC12 cells, lacking CYP2E1, were quite resistant to cytotoxicity induced by AAP and other CYP2E1 substrates. From these studies, it was concluded that the metabolism of AAP by CYP2E1 was required for toxicity. A similar conclusion was drawn in experiments using knockout mice deficient in the *CYP2E1* gene (Lee et al., 1996a).

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ABBREVIATIONS: AAP, acetaminophen; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; MAP, mitogen-activated protein; JNK, c-Jun NH_2-terminal protein kinase; ERK, extracellular signal-regulated protein kinase; PI-3K, phosphatidylinositol-3 kinase; P450, cytochrome P450; DAPI, 4,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; YH439, isopropyl 2-(1,3-dithioetane-2-ylidene)-2-[*N*-(4-methylthiazol-2-yl) carbamoyl]acetate; Z-DEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide; RT-PCR, reverse transcriptase-polymerase chain reaction; wt, wild type; SEK1, stress-activated protein kinase kinase 1; SEK1-KR, stress activated protein kinase kinase -1 Lys→Arg mutant; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair; MEKK1, mitogen-activated protein kinase kinase kinase 1; NDMA, *N*-nitrosodimethylamine.

Many distinct metabolites can be produced from AAP metabolism. Among the reactive metabolites produced by CYP2E1 and other P450 isozymes, *N*-acetyl-*p*-benzoquinoneimine (NAPQI) is the most reactive electrophilic metabolite. In therapeutic doses, NAPQI is efficiently detoxified by cellular glutathione to a less toxic NAPQI-glutathione conjugate before excretion (Thomas, 1993). When NAPQI is produced in large amounts or is inefficiently detoxified under certain conditions, such as very low levels of glutathione caused by malnutrition and/or chronic alcoholism, it often covalently binds to various cellular proteins. NAPQI most likely causes its toxic effect by interfering with the normal cellular functions of the target proteins. In fact, there have been reports correlating the levels of NAPQI protein adducts and the severity and regional location of the tissue damage (Cohen et al., 1997). However, results from different laboratories suggest that other factors, such as lipid peroxidation, Ca^{2+} homeostasis, and reactive oxygen and nitrogen species, may also be important in the AAP-mediated toxicity, because the severity of AAP-induced damage could be reduced markedly by pretreatment with Ca^{2+} antagonists (Ray et al., 1993) or macrophage inactivators such as gadolinium chloride (Michael et al., 1999), respectively. In addition, 3-hydroxyacetanilide, a noncytotoxic analog of AAP, also produced extensive protein adducts that were qualitatively similar to those of AAP (Tirmenstein and Nelson, 1989; Myers et al., 1991). Furthermore, NAPQI protein adducts were still observed when hepatocytes were protected from AAP-induced injury by pretreatment with gadolinium chloride (Michael et al., 1999). These data suggest that the NAPQI protein adducts may not be the cause of AAP-induced damage. Therefore, the exact mechanism by which AAP or its metabolite NAPQI causes cell damage is still unclear, despite the extensive literature on AAP-induced cytotoxicity. The recent data (Michael et al., 1999) led us to investigate our hypothesis that AAP and its metabolites may directly or indirectly affect the enzymes associated with cell death and survival pathways, leading to cell death upon treatment with AAP. In fact, to our knowledge, the early signaling mechanism during AAP-induced damage has not been studied systematically.

It is known that most established cell lines, including HepG2 hepatoma cells, do not contain catalytically active P450 isoforms (Dai and Cederbaum, 1995). However, earlier studies indicated that some established cell lines seem to contain certain isoforms of P450s. For instance, C6 glioma cells contain CYP2E1, CYP1A2, and other P450 isoforms as well as NADPH-dependent P450 reductase (Geng and Strobel, 1995), although the levels of these P450 enzymes are extremely low compared with their counterparts in the liver and kidney. We hypothesized in this study that CYP2E1 or CYP1A2, expressed at a very low level in C6 glioma cells, may be catalytically active and thus can metabolize AAP to a certain extent, resulting in apoptosis or necrosis upon exposure to AAP, similar to the patterns of cell death observed when CYP2E1 cDNA-transfected cells were exposed to CYP2E1 substrates such as AAP, ethanol, and nitrosodimethylamine (Dai and Cederbaum, 1995; Holownia et al., 1997; Lin et al., 1999). Our initial data showed that C6 glioma cells died upon exposure to AAP. The current study was thus undertaken to elucidate the mechanism of AAP-induced cell death by determining the activities or the levels of the three mitogen-activated protein (MAP) kinases and other proteins associated with cell death and survival pathways. These include the stress-activated protein kinase or c-Jun N-terminal protein kinase (JNK) (Karin, 1995; Xia et al., 1995) and p38 MAP kinase (Lee et al., 1994) in cell death pathway, extracellular signal-regulated protein kinase (ERK) involved in cell proliferation and survival (Xia et al., 1995), phosphatidylinositol-3 kinase (PI-3K) and Akt phosphorylation (Sonoda et al., 1999) in cell survival pathway, proapoptotic Bax protein, Bid protein translocation and cytochrome *c* release, and caspase-3 activation (Green and Reed, 1998).

Experimental Procedures

Materials. Specific monoclonal or polyclonal antibody to JNK1, cytochrome *c*, ERK1, or p38 MAP kinase was from BD PharMingen (San Diego, CA), New England Biolabs (Beverly, MA), or Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies against Bax, Bid, and Akt protein and its substrate protein were from Santa Cruz Biotechnology (Santa Cruz, CA). 4,6-Diamidino-2-phenylindole (DAPI) was purchased from Molecular Probes (Eugene, OR). Myelin basic protein, wortmannin, 3-hydroxyacetanilide, and DMSO (tissue-culture grade) were purchased from Sigma Chemicals (St. Louis, MO). SB203580 (Lee et al., 1994) was purchased from Calbiochem (San Diego, CA). The inhibitor of *CYP2E1* gene transcription (Jeong et al., 1996) isopropyl 2-(1,3-dithioetane-2-ylidene)-2-[*N*-(4-methylthiazol-2-yl) carbamoyl]acetate (YH439) was provided by Dr. J. W. Lee (Yuhan Research Center, Yuhan Corporation, Gunpo-si, Kyunggi-Do, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) was from Molecular Probes. Z-DEVD-FMK and Ac-DEVD-AMC were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Cell culture media, antibiotics, LipofectAMINE, and TRIzol were purchased from Invitrogen (Carlsbad, CA). Other reagents not listed here were the same as those described elsewhere (Jeong et al., 1996; Soh et al., 2000).

Cell Culture. Stocks of C6 glioma cells were purchased from American Type Culture Collection (Manassas, VA) and grown on plastic microtiter plates or culture dishes in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum and antibiotics (100 units/ml penicillin G, 100 μg/ml streptomycin, and 100 units of fungizocin) at 37°C in 5% CO_2 and 95% air in a humidified incubator.

Measurement of AAP-Induced Cytotoxicity. C6 glioma cells or neuro-2A cells (2×10^4 cells/well) were grown in 96-well microtiter plates for 2 days in regular serum-containing media. Varying concentrations of AAP (diluted in DMSO, 0.05% as a final concentration) were added to the culture media. After C6 glioma cells were exposed to AAP for indicated times, AAP-containing media were aspirated from the plates, and each well was washed twice with 1× phosphate-buffered saline. The viability of remaining C6 glioma was determined as described in the protocol supplied with the CellTiter 96 NonRadioactive Cell Viability Assay Kit (Promega, Madison, WI) by using MTT as a substrate. AAP-induced cytotoxicity was also determined by measuring lactate dehydrogenase (LDH) activity in the supernatant fractions using the Cytotoxicity Detection Kit (Roche Molecular Biochemicals, Summerville, NJ).

Immunocomplex Kinase Activity Assay. C6 glioma cells treated with AAP for indicated times were harvested and homogenized in ice-cold lysis buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 0.5 mM DTT, 12.5 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, and 1 μg/ml leupeptin. Cell debris and particulate fractions were removed by centrifugation at 14,000*g* for 10 min at 4°C. The activity of JNK1, ERK, or p38 MAP kinase in the soluble fraction (300 μg per reaction) was measured using a published method (Soh et al., 2000). The assay procedure included immunoprecipitation using the respective antibody (a mouse monoclonal antibody to JNK1, a polyclonal antibody to ERK1, or a polyclonal antibody to p38 MAP kinase). The kinase reaction buffer contained 20 mM HEPES, pH 7.4, 1 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na_3VO_4, 0.05 mg/ml substrate protein, 20 μM ATP, and 2 μCi [γ-^{32}P]ATP. Recombinant activator of transcription factor-2 protein (Santa Cruz Biochemicals) was used as a substrate protein for JNK1 and myelin basic protein for both ERK and p38 MAP kinase. Akt phosphorylation was measured using [γ-^{32}P]ATP by a published method (Sonoda et al., 1999). The reaction was initiated by the addition of radiolabeled ATP to the reaction mixture. After incubation for 30 min at 30°C, the enzyme reaction was stopped by 1× SDS sample buffer, and the entire reaction mixtures were subjected to electrophoresis on 12% SDS-polyacrylamide gels followed by autoradiography with intensifying screens.

Analysis of DNA Fragmentation. DNA fragmentation in C6 cells was measured using a published method (Ray et al., 1993) with the following modifications. Briefly, C6 glioma cells, treated with 5 mM AAP for indicated times, were washed with 1× phosphate-buffered saline and harvested with plastic scrapers. Cells (5×10^6 cells per 150-mm culture dish) were lysed in 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% Nonidet P-40 for 30 min on ice. This lysate was then centrifuged at 1500*g* for 5 min at 4°C to separate the fragmented DNA (supernatant) from the intact chromatin (pellet). The supernatant was incubated with RNase A (5 μg/ml) for 1 h at 37°C, followed by incubation for 2 h at 50°C with proteinase K (200 μg/ml) and 1% SDS. After extraction of the supernatant with phenol/chloroform/isoamyl alcohol (25:24:1) (saturated with 10 mM Tris, pH 8.0, and 1 mM EDTA), fragmented DNA was precipitated with 100% cold ethanol and 0.5 M ammonium acetate and subsequently air-dried. The DNA sample was dissolved in 10 mM Tri–HCl, pH 8.0, containing 1 mM EDTA, mixed with 6 volumes of DNA loading buffer (40% sucrose in 50 mM EDTA/0.25% bromphenol blue), and then loaded onto 1.8% agarose gel containing 0.2 μg/ml ethidium bromide. Electrophoresis was conducted in the running buffer (90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0) at 5 V/cm.

Transient Expression of Transfected cDNAs. The respective cDNA construct for JNK1 wt, SEK1 wt, JNK1-KR, or SEK1-KR dominant-negative mutant (Soh et al., 2000), was transfected into C6 cells (60–70% confluence), using LipofectAMINE reagent according to the manufacturer's instructions. Efficiency of transfection was determined by the percentage of immunostained cells over the total number of C6 cells fixed with 4% paraformaldehyde, pH 7.4, for 30 min. Immunostaining of fixed cells was performed by step-wise incubation with a primary antibody against the respective protein of SEK1 or JNK protein followed by a secondary antibody kit (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA).

Treatment of C6 Glioma Cells with Various Agents. C6 glioma cells, grown to 60 to 70% confluence in culture dishes (for caspase-3 assay) or 96-well microtiter plates (for cell viability assay), were pretreated with SB203580 (5 or 10 μM) for 1 h, wortmannin (5 or 10 μM) for 3 h, Z-DEVD-FMK (25 or 50 μM) for 3 h, or YH439 (10 μM) for 16 h before and during treatment with 5 mM AAP for an additional 18 to 48 h for cell viability assay. Whole homogenates from C6 glioma cells were subjected to the measurement of caspase-3 or JNK activity.

Measurement of Caspase-3 Activity. C6 glioma cells were harvested by centrifugation immediately after AAP treatment for the times indicated in Fig. 4C. Cell pellets were lysed for 60 min at 4°C in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, and 1% Triton X-100. Whole-cell homogenates (50 μg/protein) were used to determine caspase-3 activity by a method described previously (Lawson et al., 1999), measuring fluorescence generated from proteolytic cleavage of 20 μM Ac-DEVD-AMC, a fluorescent peptide substrate. Fluorescence was measured with a fluorescence plate reader (Cytoflor 2300; Millipore Corporation, Bedford, MA), excitation at 360 nm and emission at 465 nm.

Preparation of RNA and RT-PCR Analyses. Total cellular RNA was prepared from C6 glioma cells pretreated with DMSO (vehicle control) or YH439 for 16 h using the method described previously (Soh et al., 1996). Purity and recovery of RNA were determined by measuring absorbance at 260 and 280 nm. Total RNA (1 μg each) was subjected to reverse transcription followed by PCR using a kit (Promega). The oligonucleotide sequences of specific primers for rat CYP2E1 mRNA were the following: sense primer, 5′-TCT GAG GCT CAT GAG TTT GT-3′; and antisense primer, 5′-AGC AGA CAG GAG CAG AAA CA-3′. The oligonucleotide sequences of specific primers for rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA were the following: sense primer, 5′-CCA TGG AGA AGG CTG GGG-3′; and antisense primer, 5′-CAA AGT TGT CAT GGA TGA CC-3′. The amplified DNA fragments were subjected to agarose gel electrophoresis and visualized under UV illumination.

Statistical Analysis. Analyses for all experimental data shown were repeated at least three times, unless otherwise indicated. The data were analyzed by Mann-Whitney *U* test, and $p < 0.05$ was considered significant.

Results

Cytotoxic Effect of AAP on C6 Glioma Cell Death. To verify AAP-induced cell toxicity, we examined the changes in cell morphology after AAP exposure. Figure 1A represents changes in cell morphology after AAP treatment. Under DMSO-treated control conditions, C6 cells seemed healthy

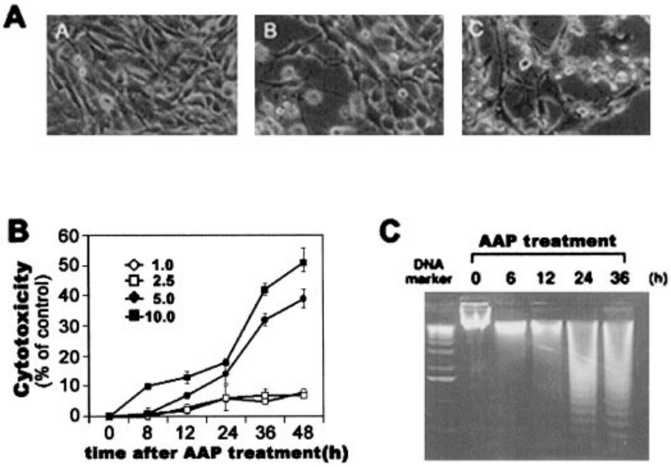

Fig. 1. Time- and AAP concentration-dependent change in cell morphology, apoptosis, and DNA fragmentation of C6 glioma cells. A, C6 glioma cells grown in culture dishes were treated with DMSO (vehicle control, left) or 5 mM AAP for 24 h (middle) and 48 h (right). Cell morphology was examined by phase-contrast microscopy under 200× magnification. B, time- and AAP concentration-dependent cell death rates were measured by the LDH release assay after C6 cells were exposed for indicated times to the following AAP concentrations: ○, 1 mM; □, 2.5 mM; ●, 5 mM; and ■, 10 mM. Each point represents the average of five determinants. C, DNA fragments of C6 cells treated with 5 mM AAP for indicated times were determined by the method as described under *Materials and Methods*.

and normal. After exposure to 5 mM AAP for 24 and 48 h, C6 cells exhibited the characteristic features of cell shrinkage, rounding, and partial detachment and demonstrated the lobulated appearance of apoptotic cells (Fig. 1A).

Direct cytotoxic effects of AAP on C6 glioma cells were then determined by measuring the release of LDH. A significant portion of cells died in concentration- and time-dependent manners upon exposure to AAP for up to 48 h (Fig. 1B). In general, the rates of cytotoxicity significantly increased at higher concentrations of AAP and longer exposure time. For instance, approximately 39 and 51% of C6 glioma cells died upon exposure to 5 and 10 mM AAP for 48 h, respectively, whereas less than 10% of C6 glioma cells died at 1 and 2.5 mM AAP under our experimental conditions. Similar levels of AAP-induced cell death were observed by the MTT reduction assay (data not shown). AAP also caused time-and dose-dependent apoptosis of neuro-2A neuroblastoma cells (data not shown).

To verify the mode of cell death, we determined the rate of DNA fragmentation for C6 glioma cells treated with 5 mM AAP for different times (Fig. 1C). A typical pattern of DNA fragmentation, a hallmark of apoptosis, was not observed within 6 and 12 h after AAP treatment. Absence of DNA fragmentation at or before 12 h post-AAP treatment (Fig. 1B) might result from a low level of cell death. However, after 24 and 36 h of treatment, DNA fragmentation was evident, indicating that AAP caused apoptosis of C6 glioma cells. Apoptosis was also verified by staining C6 glioma cells with DAPI, a dye sensitive for apoptosis. Without AAP treatment, the nuclei of control cells showed uniform staining, indicating that cells were healthy and nuclei were intact. In contrast, after AAP treatment for 36 h, approximately one half of the C6 glioma cells exhibited typical apoptotic characteristics, such as nuclear condensation as determined by staining with DAPI or Hoechst 33342 (data not shown), consistent with the DNA fragmentation data (Fig. 1C). However, some dead cells were also stained with trypan blue, suggesting that AAP could cause necrosis as well. It is particularly true that more cells died by necrosis after longer incubation time and high concentrations of AAP (>10 mM). Therefore, AAP caused cell death by apoptosis as well as by necrosis.

Dose- and Time-Dependent Selective Activation of JNK by AAP. To investigate the mechanism for AAP-induced cell death of C6 glioma cells, we studied the time- and AAP concentration-dependent effects on the catalytic activities of MAP kinases involved in early signal transduction: JNK, p38 MAP kinase, and ERK. Uniform levels of the target MAP kinase proteins and their respective substrate proteins during the assay were verified. As shown in Fig. 2A, JNK activity was slightly increased by 1 and 2.5 mM AAP, whereas it was maximally (5.6-fold) activated by 5 mM AAP. JNK activity was increased 3.8-fold by 10 mM AAP, possibly because of the rapid necrosis of C6 cells. We chose to use 5 mM AAP for our subsequent experiments because this concentration led to the maximal activation of JNK (our results) and caused apoptosis of HepG2 cells transfected with CYP2E1 cDNA, as reported previously (Dai and Cederbaum, 1995). AAP treatment activated JNK by 5.3-fold within 15 min (Fig. 2B). The elevated level of JNK persisted up to 4 h before it returned to the basal level at 8 h. However, the activities of ERK and p38 MAP kinase, which were low before treatment, remained unchanged throughout the AAP treatment. The presence of these proteins in C6 cells was confirmed by immunoblot analyses using antibody against ERK or p38 MAP kinase (Fig. 2B, bottom). The absence of activation (phosphorylation) of p38 MAP kinase by AAP was also verified using another substrate activator of transcription factor-2 in the assay and by immunoblot analysis using a specific antibody against phospho-p38 MAP kinase (data not shown). The transient and selective activation of JNK by AAP in C6 cells was also observed in the liver of mice treated with AAP (data not shown). In addition, AAP exposure also increased the phosphorylation (activation) of SEK1, the immediate upstream kinase of JNK, as in SEK1 activation by

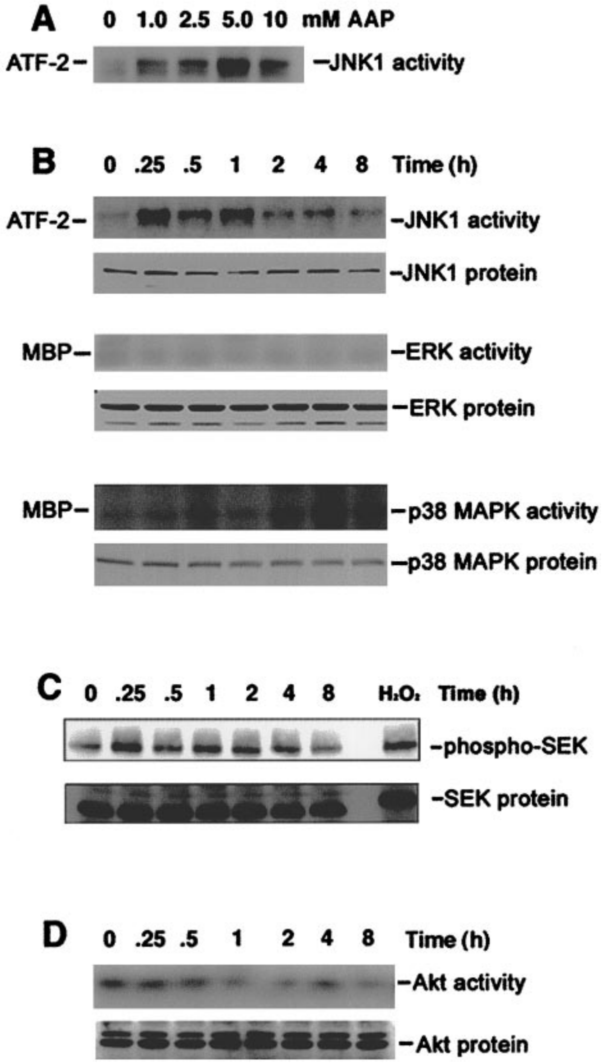

Fig. 2. Time- and AAP concentration-dependent selective activation of JNK and SEK1. A, JNK activity was measured 30 min after exposure to varying concentrations of AAP as indicated. B, catalytic activities of JNK, ERK, and p38 MAP kinase in the C6 cell extracts were determined by the immunocomplex kinase assay method, after exposure to 5 mM AAP for indicated times. Each radiolabeled protein was analyzed by SDS- polyacrylamide gel electrophoresis followed by autoradiography. Immunoblot results represent similar levels of JNK, ERK, and p38 MAP kinase used for the activity measurements. C, AAP-mediated phosphorylation (activation) and relative amount of SEK1 in C6 cells were determined by immunoblot analyses using the respective antibody against phospho-SEK1 and SEK1 protein. Elevation of phospho-SEK1 by 0.3 mM H_2O_2 for 1 h of treatment, used as a positive control, is also shown. D, AAP-mediated change in Akt phosphorylation in the C6 cell extracts was determined by the method used by Sonoda et al. (1999).

H_2O_2 treatment (Fig. 2C). In contrast, AAP did not seem to change the level of Akt phosphorylation associated with the cell survival pathway (Fig. 2D).

Effects of SB203580 and Wortmannin on AAP-Induced Cell Death. Activation of p38 MAP kinase is critically important in the cytotoxicity of certain cells because SB203580 and SB202190, specific inhibitors of p38 MAP kinase, can prevent damage caused by the withdrawal of growth factors (Lee et al., 1994) and after treatment with genotoxic stress by chemotherapeutic agents (Sanchez-Prieto et al., 2000). To test the possible role of p38 MAP kinase in AAP-induced toxicity, we pretreated C6 glioma cells with SB203580 before AAP exposure. Pretreatment with either 5 or 10 μM SB203580 did not significantly change the percentage of C6 cells undergoing apoptosis caused by 5 mM AAP (Fig. 3). This result, together with our data (Fig. 2B), strongly indicates that p38 MAP kinase is not involved in AAP-mediated cell death of C6 glioma cells.

Wortmannin is a specific inhibitor of PI-3K and was shown to inhibit the cell survival induced by growth factors (Ui et al., 1995). A recent study suggests that ethanol, another CYP2E1 substrate, can cause apoptosis of neuronal cells by inhibiting the insulin receptor-mediated PI-3K activity while elevating the levels of p53 and phospho-JNK (de la Monte et al., 2000). It is possible that AAP-induced cell death results from the potential inhibition of PI-3K by AAP. We studied, therefore, whether wortmannin pretreatment altered the rate of AAP-induced cell death. As shown in Fig. 3, wortmannin at 5 or 10 μM did not affect the degree of toxicity caused by 5 mM AAP. This result, together with the data in Fig. 2D, established that AAP causes apoptosis mainly through activating the JNK-related cell death pathway without inhibiting the PI-3K and Akt phosphorylation pathway associated with cell survival.

Increases in Bax Protein Level and Cytochrome *c* Release. Recent data indicate that the proapoptotic protein Bax or Bad interacts with antiapoptotic Bcl-2 or Bcl-XL protein in the mitochondria and suppresses the antiapoptotic function of Bcl-2, ultimately leading to cell death (Yang et al., 1995). This pattern of cell death is accompanied by the release of the mitochondrial cytochrome *c* into the cytosol, followed by activation of various caspases, which execute the cell death processes (Green and Reed, 1998). In addition, Eilers et al. (1999) recently reported that elevated c-Jun protein with phosphorylation at its N terminus can promote neuronal cell death by up-regulating the expression of either Bax or Bad protein, which stimulates the release of cytochrome *c*. We therefore studied the levels of Bax protein and cytochrome *c* release during AAP-induced cell death. Immunoblot analysis using a specific antibody against Bax or cytochrome *c* revealed that Bax protein level started to increase 8 h after AAP treatment. The elevated level of Bax persisted until 36 h after treatment (Fig. 4A). However, we could not detect any considerable change in the level of Bcl-2 in C6 cells after AAP exposure (data not shown). The level of cytochrome *c* in the mitochondria decreased in a time-dependent manner. Up to 12 h after AAP treatment, there was little change in cytochrome *c* release. These data may indicate a caspase-3–independent cell death mechanism. However, 24 h after AAP treatment, the cytochrome *c* level in the mitochondria started to decrease although its level in the cytosol increased at this time point (Fig. 4B), indicating the involvement of Bax pro-

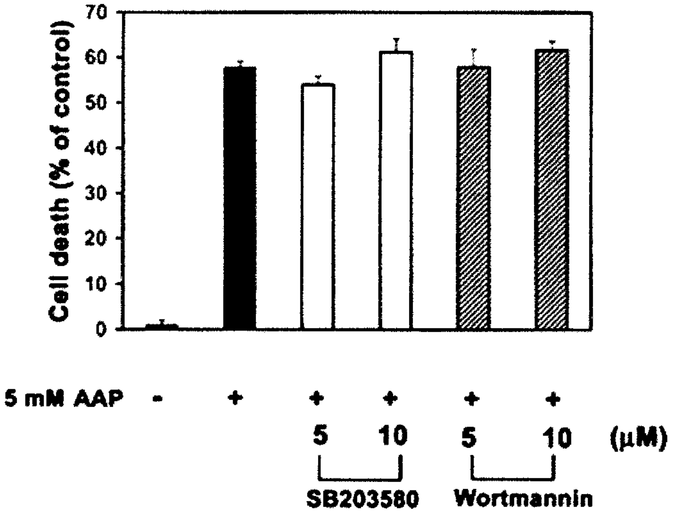

Fig. 3. Effects of SB203580 and wortmannin on AAP-induced cell death. C6 cells grown in microtiter plates were pretreated with DMSO (control), SB203580 for 1 h, or wortmannin at 5 and 10 μM for 3 h before treatment with 5 mM AAP for an additional 48 h. Cell viability was then determined with the use of the MTT viability assay.

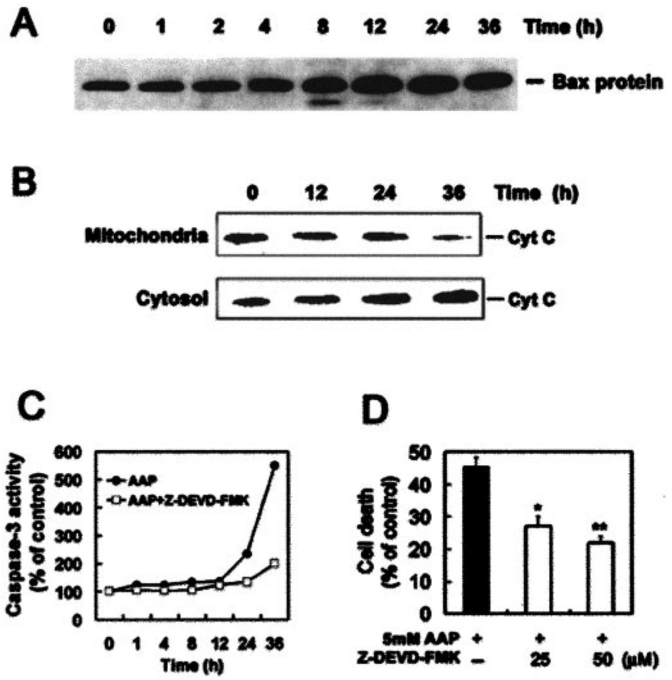

Fig. 4. AAP-induced changes in Bax, cytochrome *c* release, caspase-3 activity, and apoptosis. A, whole cell homogenates (100 μg/well), prepared from AAP-treated C6 cells for different times, were subjected to immunoblot analysis using the antibody against Bax protein. B, the mitochondrial and cytosolic fractions, prepared from AAP-treated C6 cells for different times, were subjected to immunoblot analysis using the antibody against cytochrome *c*. C, C6 glioma cells were pretreated with DMSO (vehicle control, ●) or 25 μM Z-DEVD-FMK (□) for 3 h before treatment with 5 mM AAP for additional incubation times as indicated. Whole-cell homogenates (50 μg of protein/sample) were used to measure the activity of caspase-3 using 20 μM Ac-DEVD-AMC as a fluorescent substrate. D, C6 cells, grown in microtiter plates for 36 h, were exposed to DMSO (vehicle control) or 25 or 50 μM Z-DEVD-FMK for 3 h before treatment with 5 mM AAP for an additional 48 h. Cell viability was then determined by the MTT viability assay. The results represent an average of five different determinations. Significantly different (*$p < 0.01$ and **$p < 0.001$) from the AAP-treated sample.

tein and cytochrome *c* release during the AAP-induced apoptosis.

Time-Dependent Activation of Caspase-3 upon AAP Treatment. Activation of caspases by cytochrome *c* is a key event during apoptosis caused by various toxic agents and removal of serum growth factor, although caspase-independent apoptosis mechanisms also exist in some cell systems (Stefanis et al., 1999). In addition, recent data revealed that caspase-3 activation was ruled out in the AAP-induced hepatic damage, but it was involved in the Fas receptor (CD 95)-induced hepatic damage in mice (Lawson et al., 1999). To confirm this result and determine whether caspase is activated after cytochrome *c* release (Fig. 4B), we measured the change in caspase-3 activity in C6 glioma cells after AAP treatment. As shown in Fig. 4C, caspase-3 activity was detectable but relatively low, similar to the activity of the basal control level, up to 12 h after AAP treatment. However, its activity markedly increased (approximately 5.7-fold) between 24 and 36 h after treatment. Pretreatment of C6 cells with a specific caspase-3 inhibitor, Z-DEVD-FMK, at 25 and 50 μM significantly reduced the rate of AAP-induced caspase-3 activation (Fig. 4C) as well as the cell death rate (Fig. 4D). These results demonstrate that cytochrome *c* release and subsequent activation of caspase-3 are important in executing AAP-mediated cell death of C6 glioma cells.

Critical Role of the JNK-SEK1 Pathway in AAP-Induced Toxicity. To investigate the critical role of the selective JNK activation in AAP-induced toxicity, C6 cells were transiently transfected with the cDNA for JNK wt, SEK1 wt, JNK-KR, or SEK1-KR dominant-negative mutant, followed by measurements of JNK activity and relative cell death rates upon AAP treatment. The transfection efficiency of each cDNA ranged from 8 to 13% of total C6 glioma cells, and very little JNK was activated in the absence of AAP treatment. Consistent with the data in Fig. 2, AAP treatment significantly increased the JNK activity in C6 cells transfected with the pcDNA vector alone (Fig. 5A, lane 2), compared with the DMSO-treated control (lane 1). In this system, transfection of the wild-type JNK (lane 3) or SEK1 cDNA (lane 5) did not further increase the JNK activity over the AAP-induced JNK activation, suggesting the maximum activation of JNK by AAP alone (lane 2). However, transfection of the cDNA for the JNK-KR or SEK1-KR dominant-negative mutant markedly decreased the JNK activity after exposure to 5 mM AAP (lanes 4 and 6), compared with their respective counterpart. The expression of transduced JNK (data not shown) or SEK1 protein was verified by immunoblot analysis (Fig. 5A). These results also support that AAP increases the JNK activity through the SEK1-JNK pathway.

The functional role of the JNK activation was investigated by measuring the AAP-induced cell death rate after C6 cells were transiently transfected with the same set of DNA constructs and then exposed to 5 mM AAP for an additional 36 h before the cell viability assay. Only a small fraction of C6 cells (<5%) died in DMSO vehicle control when cells were transfected with the pcDNA vector alone (Fig. 5B, lane 1). Approximately 48% of C6 cells died after AAP exposure (lane 2). In contrast, 52 or 57% of cells died when cDNA for JNK wt or SEK1 wt was transfected and then exposed to AAP (lanes 3 and 5). However, the rate of apoptosis was significantly reduced by transfecting cDNA for the dominant-negative mutant, JNK-KR (28%, lane 4) or SEK1-KR (31%, lane 6), compared with their counterparts, JNK wt or SEK1 wt. These data clearly establish a critical role of JNK and SEK1 activation in AAP-induced cell death.

Different Effects of AAP and 3-Hydroxyacetanilide on JNK Activity and Cell Death Rate. It was shown that a regiospecific analog of AAP 3-hydroxyacetanilide did not cause liver damage in animals, despite its binding to various proteins (Tirmenstein et al., 1989; Myers et al., 1991). We therefore compared the effects of AAP and 3-hydroxyacetanilide on JNK activation and the cell death rate. As shown in Fig. 6A, 3-hydroxyacetanilide (5 mM) did not activate JNK activity determined 15 and 30 min after treatment, whereas 5 mM AAP and 0.3 mM H_2O_2, used as a positive control, significantly increased the JNK activity at both times. Furthermore, less than 10% of C6 cells died at 36 and 48 h after exposure to this noncytotoxic compound, whereas approximately 30 to 50% cells died upon AAP treatment (Fig. 6B). These in vitro data are consistent with the noncytotoxic effect of 3-hydroxyacetanilide observed in animals (Tirmenstein et al., 1989; Myers et al., 1991). These data further support an

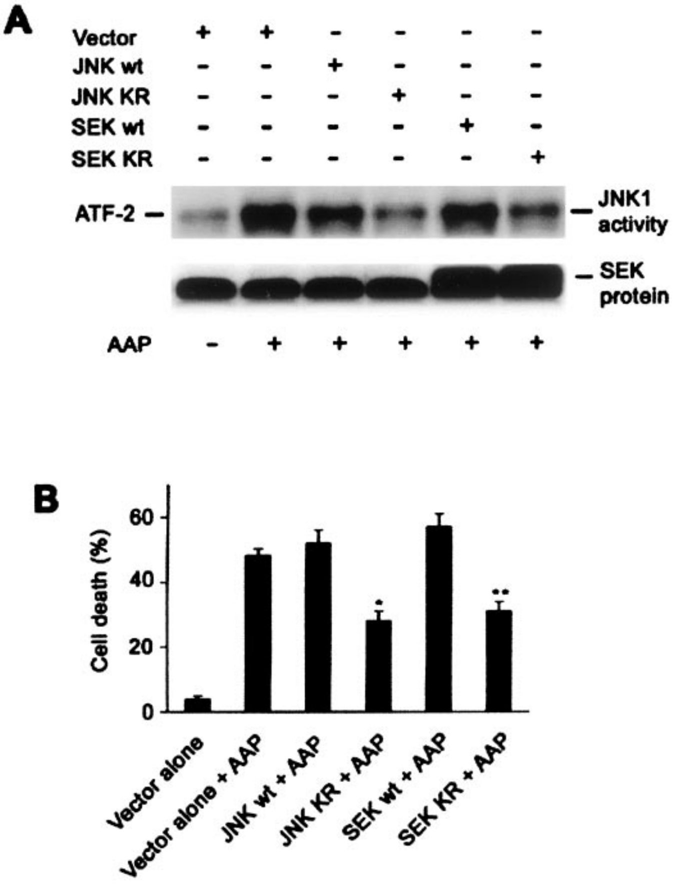

Fig. 5. Critical role of the JNK activation in AAP-induced cell death. A, the expression vector containing the cDNA for JNK-HA wt, SEK1 wt, JNK-KR, or SEK1-KR dominant-negative mutant as indicated was transiently transfected into C6 cells for 48 h. Cells were then treated with 5 mM AAP for 30 min, harvested, homogenized, and subjected to measurement of JNK activity (top). The immunoblot result represents the relative level of transduced SEK1 protein (bottom). B, C6 glioma cells (2×10^4 cells/well), grown in 96-well microtiter plates, were transiently transfected with 0.125 μg of each construct as indicated and treated with 5 mM AAP for an additional 48 h. Cell viability was then measured by use of the MTT method. * and **, significantly different ($p < 0.02$) from the corresponding JNK wt and SEK1 wt, respectively.

important role of the JNK activation in AAP-induced apoptosis.

Effects of YH439 on CYP2E1 Content and Activity, AAP-Induced JNK Activation, and DNA Fragmentation. We reported previously that a synthetic compound, YH439, transcriptionally suppresses the expression of *CYP2E1* gene (Jeong et al., 1996). Therefore, we studied the effects of YH439 pretreatment on CYP2E1 mRNA, protein content, and its catalytic activity as well as JNK activity and cell death rate upon AAP exposure. YH439 treatment markedly reduced the level of CYP2E1 mRNA (770 bp amplicon by RT-PCR) (Fig. 7A, left), whereas the mRNA level (194 bp amplicon) of GAPDH, used as a negative control, did not change during YH439 treatment (Fig. 7A, right), confirming the pretranslational suppression of *CYP2E1* gene by YH439. YH439 exposure markedly reduced the CYP2E1 content determined by immunoblot analysis (Fig. 7B). The CYP2E1 activity determined by *N*-nitrosodimethylamine (NDMA) demethylase in the S-9 fraction of C6 glioma cells increased linearly for up to 60 min of incubation. The basal CYP2E1 activity (approximately 4.6 pmol HCHO produced/mg protein of S-9 fraction/60 min) was approximately one sixth of that observed for HepG2 cells (E47 cells) containing transduced CYP2E1. However, YH439 exposure also significantly reduced the CYP2E1 activity by 51% (Fig. 7C). Furthermore, YH439 pretreatment markedly reduced the AAP-induced JNK activation (phosphorylation) (Fig. 7D) and the rates of cell death determined by MTT reduction (Fig. 7E) at two time points. These data indicate that CYP2E1-dependent AAP metabolism to NAPQI and subsequent JNK activation are important for the AAP-induced cytotoxicity.

Discussion

AAP is widely used as a safe analgesic agent in therapeutic doses. However, it can cause severe hepatic and extrahepatic damage when it is used in large quantities or in combination with alcohol consumption or under fasting conditions (Thomas, 1993; Whitcomb and Block, 1994; Schiodt et al., 1997). Because AAP overdose (peak plasma concentrations ranging from 1 to 3 mM AAP) is often observed in humans (Schiodt et al., 1997) and because 5 mM AAP was used previously to demonstrate AAP-induced cell toxicity (Dai and Cederbaum, 1995; Holownia et al., 1997), a high concentration (5 mM) of AAP used in this study was not unreasonably high. Production of NAPQI protein adducts, changes in Ca^{2+} homeostasis, reduction of ATP, and reducing equivalents, including gluta-

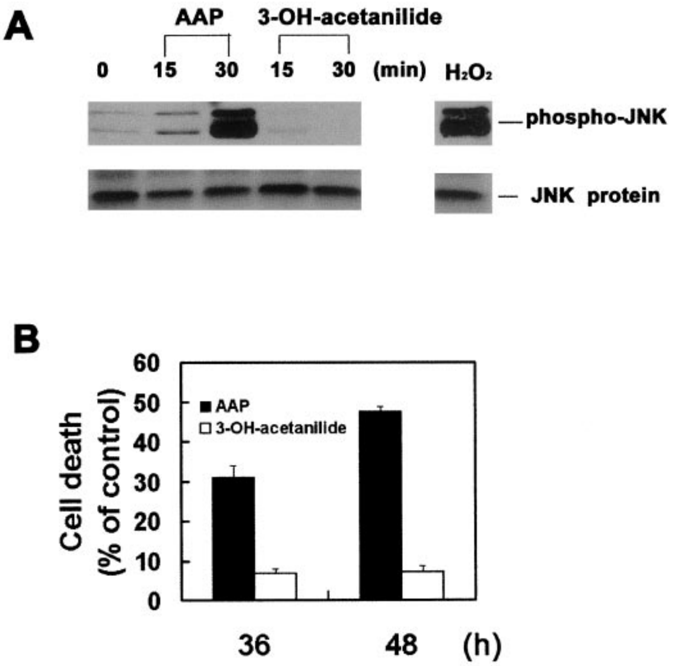

Fig. 6. Different effects of AAP and 3-hydroxyacetanilide on JNK activation and the rate of cell death. A, C6 glioma cells, grown in 150-mm culture dishes for 2 days, were exposed to 5 mM AAP or its nontoxic analog 3-hydroxyacetanilide for 15 and 30 min before JNK1 activity assay. Immediately after treatment, cells were harvested and cell extracts were prepared. JNK1 proteins in different cell extracts were immunoprecipitated with the antibody against JNK1 protein. The immunoprecipitated JNK protein was resolved by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis using the specific antibody against phospho-JNK1 protein. Phosphorylation (activation) of JNK1 by 0.3 mM H_2O_2 for 60 min treatment is also shown. B, C6 glioma cells, grown in 96-well microtiter plates for 2 days, were exposed to 5 mM AAP (■) or its nontoxic analog, 3-hydroxyacetanilide (□) for an additional 36 and 48 h before the MTT cell death assay.

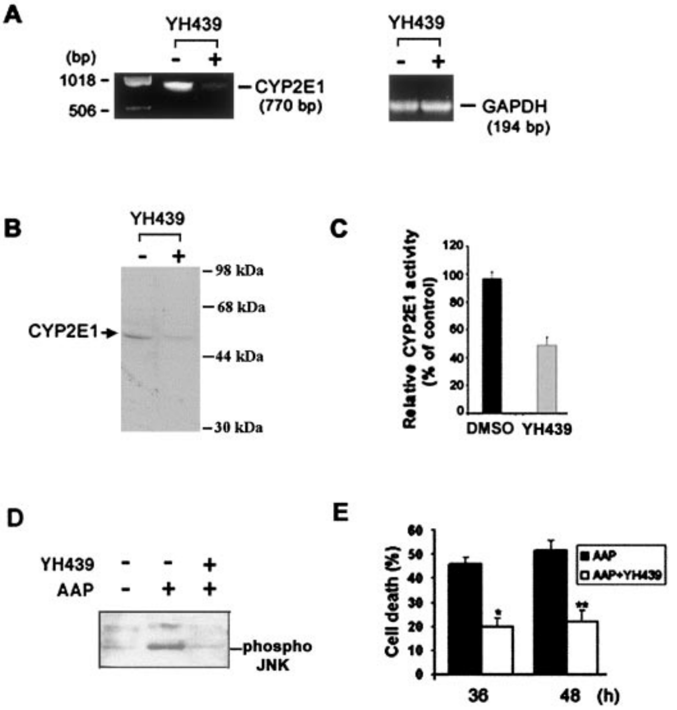

Fig. 7. Effects of YH439 on CYP2E1 mRNA level, content and catalytic activity, AAP-induced JNK activation, and cell death rate. A, total RNA, prepared from C6 cells treated with DMSO (vehicle control) or 10 μM YH439 for 16 h, were subjected to RT-PCR analysis using two different sets of specific primers for *CYP2E1* (left) and GAPDH (right). B and C, CYP2E1 content and activity were determined by immunoblot analysis (0.5 mg protein/well) (B) and NDMA demethylase activity (C), respectively, for the S-9 fractions of C6 glioma cells, as described previously (Jeong et al., 1996). The enzyme reaction mixture contained 50 mM Tris-HCl, pH 7.4, 1 mM NDMA, 10 mM $MgCl_2$, 50 mM KCl, and 5 mg of S-9 fractions of C6 cells. The reaction was terminated after incubation for 60 min at 37°C, and the amount of HCHO produced was measured spectrophotometrically at 412 nm. D, C6 glioma cells grown in culture dishes were pretreated with DMSO or 10 μM YH439 for 16 h before AAP treatment. Cells were harvested immediately after exposure to DMSO or 5 mM AAP for an additional 30 min, and whole-cell extracts were prepared. JNK1 activation was then determined by immunoblot analysis using the specific antibody against phospho-JNK1 protein. E, C6 glioma cells, grown in microtiter plates for 36 h, were then exposed to 10 μM YH439 for 16 h before and during treatment with 5 mM AAP for an additional 36 or 48 h. Cell death rate was determined by MTT reduction.

thione, elevated levels of oxidative stress, and lipid peroxidation, have been proposed to account for AAP-induced toxicity (Ray et al., 1993; Cohen et al., 1997; Michael et al., 1999). Despite numerous reports describing NAPQI protein binding and subsequent tissue damage, the signaling cascade through which AAP or NAPQI causes cell or organ damage is still unclear. To our knowledge, the early signaling events during AAP-induced apoptosis have not been studied systematically.

It is conceivable that AAP-induced cell damage could result from interference with the enzymes or proteins involved in the cell survival pathway, as reported recently for the ethanol-induced neuronal cell death (de la Monte et al., 2000). These proteins include focal adhesion kinase, PI-3K activation, Akt phosphorylation, and phosphorylation of proapoptotic Bad protein (Sonoda et al., 1999). Phosphorylated Bad no longer binds antiapoptotic Bcl-2 or Bcl-XL proteins (Zha et al., 1996) and interrupts their antiapoptotic functions, thus leading to enhanced cell survival. Few changes in the levels of ERK and Akt phosphorylation after AAP treatment along with the negligible effect of wortmannin on cell death rate clearly demonstrate that AAP induces apoptosis without inhibiting the enzymes or proteins associated with the cell survival pathway. Our data establish that AAP and/or its metabolites exert their cytotoxicity mainly by promoting the enzymes or proteins associated with the cell death pathway through the selective activation of the SEK1-JNK pathway. Our conclusion was further supported by the following: 1) direct activation of SEK1 and JNK by AAP; 2) little activation of p38 MAP kinase; 3) negative effects of SB203580; 4) results from transfection of the respective cDNA for JNK-KR or SEK1-KR dominant-negative mutant; and 5) effects of 3-hydroxyacetanilide and YH439 on JNK activation and cell death rate.

The absence of p38 MAP kinase activation by AAP is of interest because stressful conditions and several toxic agents increase JNK and p38 MAP kinase activities, often in a coordinated fashion. These stimulants include the withdrawal of serum growth factor, various proinflammatory cytokines, UV and X-ray irradiations, H_2O_2, and other cytotoxic or genotoxic chemicals (Lee et al., 1994; Karin, 1995; Xia et al., 1995; Sanchez-Prieto et al., 2000). The lack of activation of p38 MAP kinase after AAP treatment was not caused by the absence of this enzyme in C6 glioma cells, because this protein was clearly detected by our immunoblot analysis. Our current results are consistent with the selective activation of the SEK1-JNK pathway by other CYP2E1 substrates, such as 4-hydroxy-2-nonenal (Soh et al., 2000) and CCl_4 (Mendelson et al., 1996; data not shown).

It is important to determine how these CYP2E1 substrates selectively activate the JNK pathway without affecting p38 MAP kinase. Because YH439 significantly suppressed the levels of CYP2E1 mRNA, CYP2E1 content and its catalytic activity, JNK activity, and cell death rate, NAPQI or other reactive metabolites produced from CYP2E1-mediated AAP metabolism must be responsible for stimulating the JNK pathway. Therefore, it is reasonable that NAPQI and other reactive metabolites can directly activate MEKK1 and ASK1, the upstream kinases of the JNK-SEK1 pathway (Karin 1995; Xia et al., 1995). Alternatively, these reactive metabolites can affect the association/dissociation of small GTP-binding proteins such as rac and rho in the plasma membrane, resulting in the activation of the MEKK1-SEK1-JNK pathway. These reactive metabolites can also activate a phosphatase, which specifically dephosphorylates phospho-p38 MAP kinase, as demonstrated recently (Mendelson et al., 1996). Furthermore, these reactive metabolites may inhibit specific phosphatase(s) that selectively remove the phosphate group from phospho-SEK1 or phospho-MEKK1. We are currently testing these possibilities in C6 glioma cells and HepG2 cells with or without transduced CYP2E1.

Whether and how JNK activation is critically important for cell death seems to depend on the cell type, cell death agonists, the duration of JNK activation, and the cellular microenvironment (Karin, 1995; Xia et al., 1995; Mendelson et al., 1996; Soh et al., 2000). During and after JNK activation, c-Jun protein is phosphorylated and heterodimerizes with another protein, c-fos, to generate the transcription factor AP-1, as reported previously for AAP-induced hepatotoxicity (Blazka et al., 1996). The activated AP-1 then interacts with specific DNA binding sites and thereby regulates the transcription of various target genes such as Bax or Bad protein, which promotes the release of cytochrome *c* before cell death, as described recently (Eilers et al., 1999). Although we did not establish a direct relationship between the JNK activation and the increased level of Bax protein in this study, sequential elevations of JNK activity, Bax protein, cytochrome *c* release, and caspase-3 activity suggest that these proapoptotic proteins are important in AAP-induced apoptosis. It is also possible that the JNK signaling pathway may affect the phosphorylation and subsequent inactivation of the mitochondrial target proteins involved in cell survival, such as Bcl-2 and its related proteins (Maundrell et al., 1997; Yamamoto et al., 1999). In contrast, p38 MAP kinase or ERK was not as efficient as JNK in phosphorylating Bcl-2 protein (Maundrell et al., 1997). These facts support the idea that the selective JNK activation by AAP (current study) and 4-hydroxy-2-nonenal (Soh et al., 2000) may be sufficient to initiate apoptosis of the target cells. Alternatively, the JNK signaling pathway may promote the proteolytic cleavage and translocation of Bid, a proapoptotic BH3 protein of the Bcl-2 family members, to the mitochondria before cytochrome *c* release and caspase activation (Li et al., 1998). Recent data with primary embryonic fibroblasts from double knockout mice deficient in *JNK1/2* genes supported the latter case, strongly suggesting that JNK activation may directly or indirectly affect this mitochondrial death-signaling pathway including the release of cytochrome *c* and caspase activation (Tournier et al., 2000). To check whether Bid translocation was involved in AAP-induced apoptosis, we tried to determine Bid translocation from the cytosol to mitochondria by immunoblot analysis. However, we could not detect Bid translocation in the extracts of C6 cells, possibly because of a low level of Bid expression in C6 cells. Therefore, the role of Bid in AAP-induced apoptosis of C6 glioma cells is unclear. In contrast, our preliminary data revealed that Bid protein was translocated from the cytosol to mitochondria of AAP-treated HepG2 cells with transduced CYP2E1 (E47 cells kindly provided by Dr. Arthur I. Cederbaum, Mount Sinai School of Medicine, New York, NY), supporting a role of Bid translocation in AAP-induced apoptosis in another cell system. Nonetheless, our current results with C6 glioma cells clearly demonstrate the involvement of cytochrome *c* release and activation of caspase-3 by AAP treatment, contrasting with the earlier study in which caspase-3 activation was ruled out in AAP-induced liver damage (Lawson et al., 1999).

Earlier reports revealed that C6 glioma cells contain catalytically active P450 isozymes including CYP1A2, CYP1B1/2, and CYP2E1. These P450 proteins can be up-regulated by their respective inducers (Geng and Strobel, 1995). Although the expressed levels of these P450 isozymes are very low, compared with their counterparts in the liver and kidney, they may be catalytically active because of the presence of NADPH-dependent P450 reductase (Geng and Strobel, 1995). Our current data demonstrated a low but detectable level of CYP2E1 activity in C6 glioma cells and that CYP2E1 content and activity could be down-regulated by YH439 treatment. Therefore, the use of C6 glioma cells may provide multiple advantages in studying the mechanism of apoptosis caused by AAP or other toxic compounds. First, there is no need to transfect the cDNA for CYP2E1 or CYP1A2, which is usually absent in other established cell lines, including HepG2 hepatoma cells. Second, valuable time, efforts, and resources can be spared in selecting the stable transformants with the target cDNA. Third, using cultured cells, which are relatively less complex than the in vivo models, we can not only study the detailed signaling mechanisms for cell death and survival pathways but also test the potential benefits of certain compounds against chemical-induced toxicity within a short period of time and limited resources. Finally, we can always rely on the relatively constant levels of CYP2E1 expressed in culture cells despite its low level of expression. Therefore, it is possible to study the signaling mechanisms for apoptosis by other CYP2E1 substrates in these established cell lines, although C6 glioma cells may not fully reflect the AAP-induced cell damage of hepatic origins. It is possible that C6 glioma cells are sensitive to AAP-induced toxicity because of the low level of glutathione (basal level is approximately 12–13 nmol/mg protein) compared with hepatoma cells or primary hepatocytes.

Our previous data (Jeong et al., 1996) and current results established that YH439 markedly reduced the levels of CYP2E1 mRNA and activity. Although we did not determine the levels of *CYP1A1/2* in C6 glioma cells, their levels might be elevated by YH439 pretreatment, as expected from our earlier results on *CYP1A1/2* genes (Lee et al., 1996b). From the significant reductions in CYP2E1 level and AAP-induced JNK activation and cell death by YH439 pretreatment, we note that it is more likely that CYP2E1-dependent AAP metabolism is more important for producing NAPQI than the CYP1A2-catalyzed AAP metabolism. Our conclusion is consistent with previous results describing a minimal role of CYP1A2 in AAP-induced hepatotoxicity in knockout mice deficient in the *CYP1A2* gene (Tonge et al., 1998). However, because YH439 does not change CYP3A levels, the importance of CYP3A-mediated AAP metabolism in apoptosis of C6 cells could not be determined.

In conclusion, the data presented in this study demonstrate that established cell lines, such as C6 glioma cells, which express CYP2E1 in small quantity, can be used as a model to study the signaling mechanisms for cell death and survival pathways. Our data also indicate the importance of CYP2E1-dependent metabolism of AAP, whose effects could be markedly reduced by pretreatment of YH439, an inhibitor of *CYP2E1* gene transcription. Furthermore, these data establish that AAP and possibly its toxic metabolite NAPQI can cause cell damage by promoting the JNK-dependent cell death pathway without inhibiting the cell survival pathway. Therefore, our current data represent a new mechanism that is clearly different from the previously known mechanisms for AAP-induced toxicity.

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